For general laboratory use.

Roche Applied Science

DNA Isolation Kit for Mammalian Blood

Instruction Manual

Version 3, August 2004

Size: up to 10 ml mammalian blood

Cat. No. 11 667 327 001

Store at +2 to +8°C

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2. Product Description

Intended use

The DNA Isolation Kit for Mammalian Blood simplifies the rapid isolation of DNA from 1–10 ml whole blood (*e.g.*, from human, mouse, rat). The isolated DNA is suitable for many applications, including standard PCR, long PCR, sequencing, and Southern blots. In addition to the standard "DNA Isolation from 10 ml Mammalian Whole Blood" procedure, this kit can also be used for the isolation of DNA from buffy coat and lymphocyte samples.

Isolation of DNA from whole blood can be difficult because blood is a complex mixture containing cells, proteins, metabolites, etc. Most of the cells (>99%) are erythrocytes (red blood cells), which lack nuclei, and therefore, possess no DNA. Only leukocytes (0.3% of total blood cells), also called white blood cells, contain nuclei and DNA. Therefore, DNA from blood must be isolated from one of three types of leukocytes: monocytes, lymphocytes (25% of the leukocyte population), or granulocytes (1).

The DNA isolated from whole blood must be free of contaminants that may interfere with such applications as PCR. For example, the presence of contaminating heme in the final DNA preparation has been shown to strongly inhibit Taq DNA polymerase during PCR (2).

The DNA Isolation Kit for Mammalian Blood provides a simple, rapid, and safe method for the effective isolation of pure DNA. The entire procedure can be completed in less than 1.5 h (plus the resuspension time), and this easy-to-use kit employs fewer steps than standard methods (*e.g.*, Ficoll-Hypaque density gradients). This simplifies the simultaneous processing of multiple samples. Safety is another key attribute of the kit, which reduces handling of blood and eliminates the need for organic extractions or chaotropic agents.

Principle

The DNA Isolation Kit for Mammalian Blood procedure relies on separation of the white blood cells from whole blood via a preferential red blood cell lysis. In the presence of a strong anionic detergent, the white blood cells are then lysed, and the proteins removed by dehydration and precipitation. The purified DNA is subsequently recovered via ethanol precipitation (3,4).

Kit contents

1. Red Blood Cell Lysis Buffer, 750 ml
2. White Blood Cell Lysis Buffer, 125 ml
3. Protein Precipitation Solution, 65 ml

Storage and stability

The kit components are stable through the control date printed on the box (24 months from date of manufacture) when stored at +2 to +8°C.

Note: A white precipitate may be observed in the White Blood Cell Lysis buffer at lower temperatures. This precipitate will not affect the function of the kit. Dissolve the precipitate by warming the solution for approximately 15 minutes in a 37°C water bath, with occasional swirling.

3. Applications

Safety precautions

Employ universal safety precautions when working with biohazardous materials. Wear lab coats, gloves, and safety glasses at all times. Properly dispose of all contaminated materials. Decontaminate work surfaces, and use a biosafety cabinet whenever aerosols might be generated.

Additionally required materials

In addition to the kit components, these additional materials will be needed for DNA isolation from mammalian blood:

- Ethanol
- 70% Ethanol
- TE Buffer, pH 8.0 (optional)
- RNase (optional)

4. DNA Isolation from 10 ml Mammalian Whole Blood

Starting material

- Use 10 ml mammalian whole blood (*e.g.*, from human, mouse, rat). When using <10 ml blood, follow the alternate procedure titled, "Optimal procedure for use with smaller quantities of blood" for modifications to this general procedure.
- If blood was stored at +2 to +8°C or –15 to –25°C, warm the blood to +15 to +25°C prior to use.
- If using human blood components, such as lymphocytes or buffy coat, follow the alternative procedure titled, "Procedural modifications for use with buffy coat or lymphocyte samples."
- Do not use blood that has been frozen and thawed more than 3 times, or yields will be significantly reduced.
- For best results, use fresh blood or blood stored for ≤3 days. Blood stored for 7 days at +2 to +8°C or ≤1 month at –15 to –25°C will result in a 10–15% reduction in yield.
- Use sodium heparin-, sodium citrate-, or EDTA-treated blood. For heparin-treated blood, heat the white cell pellet in White Blood Cell Lysis Buffer for 10 min at 65° to facilitate lysis (step 7).

Procedure

Perform all steps at +15 to +25°C unless otherwise indicated.

		(for 4 samples)	
Step	**Isolation Procedure**	**Actual time**	**Hands-on time**
1	For each blood sample to be processed, add 30 ml Red Blood Cell Lysis Buffer to a sterile 50-ml centrifuge tube. **Notes:** Use a centrifuge tube that will withstand a minimum of 900 x *g* (preferable 12,000 x *g*) and accommodate a total volume of 40 ml.	30 sec	30 sec
2	To each tube, add 10 ml mammalian blood. Cap the tube, and mix gently by inversion. **Notes:** Do not vortex!	30 sec	30 sec
3	Place the centrifuge tube on a rocking platform or gyratory shaker for 10 min. **Notes:** Alternatively, manually invert the sample periodically for 10 minutes.	10 min	30 sec
4	Centrifuge the tube at 875 x *g* for 10 min (*e.g.*, 2,000 rpm in a RT6000B Sorvall Centrifuge). **Notes:** Do not exceed centrifugation speed limit, as this will increase the difficulty of resuspending the white cell pellet (step 6).	12 min	1 min
5	Carefully pour off and properly dispose of the clear, red supernatant (indicative of complete red cell lysis). Some residual liquid should remain with the white cell pellet. **Notes:** The Red Blood Cell Lysis Buffer selectively lyses the erythrocytes, leaving the leukocytes intact. Following centrifugation, if the sample appears as a cloudy upper layer (containing plasma/leukocytes) and a red lower layer (containing erythrocytes), no red cell lysis has occurred. If this happens, do one of the following: • Repeat steps 1–4 with fresh blood, using a 15-minute incubation in step 3. • Repeat steps 1–4 with fresh blood, inverting the sample more frequently if mixing by hand. • Ensure that fresh blood has been warmed to +15 to +25°C before repeating steps 1–4.	30 sec	30 sec

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4. DNA Isolation from 10 ml Mammalian Whole Blood, continued

Step	Isolation Procedure	Actual time	Hands-on time
6	Thoroughly vortex the white pellet visible at the bottom of the tube in the residual supernatant. **Notes:** • *Vortex thoroughly*, making sure that the white cell pellet is fully resuspended in the residual supernatant. This step facilitates complete lysis of the white cell pellet during step 7. • Note that the white cell pellet will still be slightly red in color due to the presence of residual hemoglobin. The hemoglobin will be removed in subsequent steps.	1 min	1 min
7	Add 5 ml White Cell Lysis Buffer, cap the tube, and mix thoroughly by vortexing. **Notes:** • *Vortex thoroughly* to ensure the white cells are completely lysed. Following successful lysis of the leukocytes, the solution should appear clear dark red/brown with no particulate material present. To ensure this, perform an optional incubation at 37°C for 15–30 minutes, which may facilitate lysis. • For heparin-treated blood, heat the white cell pellet in White Blood Cell Lysis Buffer for 10 min at 65°C to facilitate lysis.	1 min	1 min
8	**Optional:** Add RNase* to a final concentration of 0.02 mg/ml. Mix by inversion, and incubate at 37°C for 15 minutes. **Notes:** • RNase treatment completely removes RNA contaminants from the final DNA sample, yielding pure DNA with no RNA contamination. However, if subsequent applications do not require a RNA-free sample, this step can be eliminated. • The incubation time can be extended up to 1 h at 37°C.	15.5 min (optional)	30 sec

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4. DNA Isolation from 10 ml Mammalian Whole Blood, continued

		(for 4 samples)	
Step	**Isolation Procedure**	**Actual time**	**Hands-on time**
9	Transfer the sample to a sterile 17 x 100 mm tube. **Notes:** The sample can remain in the 50 ml centrifugation tube if the 50 ml tube is capable of withstanding 12,000 x *g* centrifugal force.	30 sec	30 sec
10	Add 2.6 ml Protein Precipitation Solution to each sample. **Notes:** • *Vortex thoroughly!* (We recommend that you vortex continuously for approximately 25 seconds.) This is necessary for effective removal of protein from the sample. • Upon vortexing, a brownish protein precipitate will be clearly visible.	1 min	1 min
11	Centrifuge the sample at 12,000 x *g* (*e.g.*, 10,000 rpm in a Sorvall RC5B centrifuge) for 10 minutes. **Notes:** Samples must be centrifuged at 12,000 x *g* for a minimum of 10 minutes. Lower-speed spins will result in loose protein pellets, making it very difficult to effectively separate the proteinaceous material from the supernatant.	12 min	1 min
12	Carefully pour the supernatant, which contains the DNA, into a new sterile 50 ml centrifuge tube. Properly dispose of the protein pellet. **Notes:** The new centrifuge tube must accommodate approximately 30 ml and withstand a minimum of 900 x *g* (or 12,000 x *g* for isolation of $<1.5 \times 10^7$ cells).	30 sec	30 sec

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4. DNA Isolation from 10 ml Mammalian Whole Blood, continued

		(for 4 samples)	
Step	**Isolation Procedure**	**Actual time**	**Hands-on time**
13	Ethanol precipitate the DNA: **Notes:** • Add 2 volumes of +15 to +25°C ethanol to the supernatant from step 12. Gently mix by inversion until DNA strands precipitate out of solution and the remaining liquid is no longer cloudy. • Centrifuge the sample at 875 x *g* for 10 minutes (*e.g.*, 2,000 rpm in a Sorvall RT6000B centrifuge). Discard the supernatant. For $<1.5 \times 10^7$ cells, centrifuge at 12,000 x *g* for 10 minutes following addition of glycogen carrier. **Optional Method:** Instead of centrifugation, a sterile blunt-ended glass rod may be used to carefully remove the DNA strands from the 100% ethanol before transferring them to a new sterile tube containing cold 70% ethanol (see step 14). Swirl until DNA strands are released into 70% ethanol. **Notes:** If the number of leukocytes in the sample is less than 1.5×10^7 cells, the yield may be insufficient to see visible DNA strands falling out of solution. If this occurs, add glycogen* carrier prior to the ethanol precipitation, and increase the centrifugal to 12,000 x *g* to facilitate effective precipitation.	13 min	1 min
14	Add 3 ml cold 70% ethanol to the DNA pellet in the tube, and carefully mix the sample several times by gentle inversion. Centrifuge the sample at 875 x *g* for 5 minutes (*e.g.*, at 2,000 rpm in a Sorvall RT6000B centrifuge). Discard the supernatant. **Notes:** Do not vortex during this wash step. For samples containing $<1.5 \times 10^7$ cells, centrifuge the DNA at 12,000 x *g* for 5 minutes after the 70% ethanol wash.	8 min	1 min

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4. DNA Isolation from 10 ml Mammalian Whole Blood, continued

			(for 4 samples)
Step	**Isolation Procedure**	**Actual time**	**Hands-on time**
15	Dry the DNA pellet by placing the sample under vacuum without heat for a few minutes or until the ethanol is no longer visible. **OR** Allow the sample to air dry. **Notes:** Do not overdry the DNA pellet as this will make it much more difficult to fully resuspend the DNA.	5–15 min	0–1 min
16	To resuspend the DNA pellet, add 1 ml TE Buffer, pH 8.0, or desired buffer. Vortex thoroughly. Place samples at 65°C for 30–60 min to aid in resuspension; periodically vortex the samples. **Notes:** • Vortex thoroughly to help resuspend the DNA pellet. • For human blood samples, a 30-minute incubation at 65°C is sufficient to fully resuspend DNA samples. For samples from other mammalian species, place the samples at 65°C for 60 minutes.	1 min + resuspension (30–60 min)	1 min
17	Store samples at +2 to +8°C until use. **Notes:** If desired, samples can be accurately quantified using spectrophotometry or fluorometry.		
	Total time: ≤1.5 h + resuspension (for 4 samples) **Hands-on time:** ≤15 min		

4. DNA Isolation from 10 ml Mammalian Whole Blood, continued

Using the isolated DNA

DNA prepared using the DNA Isolation Kit for Mammalian Blood can be effectively used in multiple applications, including Southerns and PCR with either Taq DNA polymerase or Expand PCR System*,‡ products. Once quantified, use the same amount of DNA per application as you would typically use of DNA prepared with an alternative purification method.

Yields can be determined via spectrophotometry or fluorometry. Average yields are approximately 350 µg/10 ml, ranging from 200–700 µg for healthy human blood (average, 5 x 10^6 leukocytes/ml). Note that the amount of DNA recovered will vary significantly depending on the number of white cells present in the donor blood. Average yields obtained from other species:

The A_{260}/A_{280} ratio for isolated DNA samples is typically 1.7–1.9.

Species	Average yields	Yield range
Mouse	570 µg/10 ml blood	430–670 µg
Rat	580 µg/10 ml blood	350–680 µg
Dog	450 µg/10 ml blood	350–600 µg
Porcine	670 µg/10 ml blood	520–780 µg
Guinea Pig	160 µg/10 ml blood	55–295 µg

Optional procedure for use with smaller quantities of blood

By slightly adjusting the procedure detailed in the section titled "DNA isolation from 10 ml mammalian whole blood," blood samples from 1–10 ml can be processed. Follow the procedure described above with the following volume modifications:

Blood Volume (ml)	Red Blood Cell Lysis Buffer Volume: Blood Ratio† (Step 1)	White Blood Cell Lysis Buffer Volume (ml) (Step 7)	Protein Precipitation Solution Volume (ml) (Step 10)	Recommended Resuspension Volumes (µl) (Step 16)
9–<10	3:1	5.0	2.6	1000
8–<9	3:1	5.0	2.6	800
7–<8	3:1	5.0	2.6	800
6–<7	3:1	5.0	2.6	600
>5–6	3:1	5.0	2.6	600
4–5	3:1	2.5	1.3	400
3–<4	3:1	1.5	0.780	400
2–<3	3:1	1.0	0.520	200
1–<2	3:1	1.0	0.520	200

†Add 3 ml Red Blood Cell Lysis Buffer for every 1 ml whole blood

Adjust the size of the centrifuge tube as necessary to accommodate the volumes being used.

5. Procedural Modifications for Use with Buffy Coat or Lymphocyte Samples

Freshly isolated buffy coat or lymphocyte samples can be used as starting material for the purification of DNA by slightly adjusting the procedure detailed in the section titled "DNA isolation from 10 ml mammalian whole blood."

For lymphocytes

a. Isolate the lymphocyte population from 10 ml human blood, following the procedure outlined in the package insert provided with the lymphocyte separation medium you choose (*e.g.*, using standard Ficoll-Hypaque density gradients).

b. Wash the lymphocyte population twice with sterile 1x PBS prior to use.

c. At this point, an aliquot may be removed to determine cell counts.

d. Once the lymphocyte cell pellet is prepared, proceed directly to step 7 of the section titled "DNA isolation from 10 ml mammalian whole blood," modifying the procedure to add 2.5 ml White Blood Cell Lysis Buffer per sample.

   Adjust the subsequent steps accordingly (*e.g.*, use 1.3 ml Protein Precipitation Solution in step 10). Resuspension of the DNA in 500 µl TE, pH 8.0, is recommended as a starting point. **Note:** Average yields range from 75–300 µg/1.1 - 4.2 x 10^7 cells.

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5. Procedural Modifications for Use with Buffy Coat or Lymphocyte Samples, continued

For buffy coat

a. Prepare the buffy coat from 10–20 ml human blood by placing the sample at +15 to +25°C for 30 minutes or at +2 to +8°C overnight to allow the phases to separate.

   Alternatively, the blood may be centrifuged at 1,300 x *g* for 15 minutes at +15 to +25°C. The buffy coat is the interface between the plasma-containing upper phase and erythrocyte-containing lower phase.

b. Once phase separation has occurred, carefully remove and discard the upper plasma phase with a sterile Pasteur pipette, exposing the buffy coat layer.

c. Transfer the buffy coat to a sterile 17 x 100 mm tube capable of withstanding a centrifugal force of 12,000 x *g*.

   Be careful not to remove any of the erythrocyte layer with the buffy coat.

   At this point, an aliquot may be removed to determine cell count. If the total number of leukocytes isolated is less than 1.0×10^7, the procedure will not work effectively. Obtain more blood sample, and start the procedure again.

d. To the isolated buffy coat, add 5 ml sterile 1x PBS, mix gently by inversion, and centrifuge at 875 x *g* for 10 minutes at +15 to +25°C. Discard the supernatant.

e. Once the white cell pellet is obtained, proceed directly to step 7 of the section titled "DNA isolation from 10 ml mammalian whole blood," modifying the procedure to add 1.5 ml White Blood Cell Lysis Buffer per sample. Adjust the subsequent steps accordingly (*e.g.*, use 780 µl Protein Precipitation Solution in step 10). Resuspension of the isolated DNA in 300 µl TE, pH 8.0, is recommended as a starting point. **Note:** Average yields range from 35–105 µg/$1.1–2.3 \times 10^7$ cells.

6. Quality Control

Absence of DNase contamination	Each lot of the DNA Isolation Kit for Mammalian Blood is tested to ensure the absence of DNase contamination. The Red Blood Cell Lysis Buffer, White Blood Cell Lysis Buffer, and Protein Precipitation Solution are each incubated with 1 µg pBR322 DNA for 6 hours at 37°C. The DNA is then visualized by electrophoresis on an agarose gel and compared to a positive control to determine if any linear or nicked plasmid DNA is visible.
DNA isolation and amplification	Each lot of kits is function tested for the ability to purify DNA from human whole blood, followed by specific amplification of a 4.8 kb tPA fragment via PCR with the Expand Long Template PCR System*,‡. The 4.8 kb tPA product is visualized by electrophoresis on an agarose gel, and two samples are compared with a positive control of human genomic DNA to determine if the same size amplification product is obtained. An intense, single 4.8 kb tPA band is visible.

7. Appendix

7.1 References

1. *Molecular Biology of the Cell* (1989) Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. p. 973–976, Garland Publishing.
2. *PCR Technology: Principles and Applications for DNA Amplification* (1989) p. 31–38, Henry A. Erlich, ed., Stockton Press.
3. Miller, S.A., Dykes, D.D. and H.F. Polesky (1988) *Nucleic Acids Research* **16**(3): 1215.
4. Lahiri, D.K. and Schanbel, B. (1993) *Biochemical Genetics* **31**(7/8): 321-329.

7.2 Troubleshooting Guide

Problem	Remedy
No red blood cell lysis	If the sample appears as a cloudy upper layer (containing plasma/ leukocytes) and a red lower layer (containing erythrocytes) following centrifugation, no red cell lysis has occurred: • Repeat steps 1-4 with fresh blood, using a 15-minute incubation in step 3. • Repeat steps 1-4 with fresh blood, inverting the sample more frequently if mixing by hand. • Be certain to warm the blood to +15 to +25°C, and repeat steps 1–4.
Incomplete white blood cell lysis	Evident by particulate material present in sample following vortexing (see step 7) • Prior to addition of White Blood Cell Lysis Buffer, vortex the white cell pellet thoroughly, making sure that the white cell pellet is fully resuspended in the residual volume. This step facilitates complete lysis of the white cell pellet in step 7. If this is not done, it will be very difficult to completely resuspend the white cell pellet in White Blood Cell Lysis Buffer. • Vortex the sample thoroughly following addition of the White Blood Cell Lysis Buffer to ensure that the white cells are completely lysed. • Increase the volume of White Blood Cell Lysis Buffer to accommodate a larger number of white cells. If too many cells are present, the solution will become very viscous, and the cells will clump. • To facilitate complete lysis, samples may be incubated at 37°C for 15–30 minutes.
No protein pellet is observed following protein precipitation	• Mix the sample thoroughly by vortexing after addition of the Protein Precipitation Solution. Recommendation: Vortex continuously for approximately 25 seconds. • If the leukocyte number is small ($<1 \times 10^7$ cells), the protein pellet may be visible as a small, tan/brown or clear pellet. When using lymphocytes or buffy coat starting material, the protein pellet will be clear. • To ensure effective pelleting of protein, the samples must be spun at 12,000 x *g* for a minimum of 10 minutes. Lower-speed spins will result in loose protein pellets, which make it very difficult to effectively separate the protein from the supernatant.
DNA resuspension: samples are slow to rehydrate	• Samples were overdried prior to resuspension. Do not exceed 5 minutes of drying time under vacuum, and do not use heat when drying. To avoid these problems, air dry the samples, which helps to reduce overdrying. This method requires a longer time to complete. • Heat to 65°C to aid resuspension. Do not exceed 1 h incubation time at 65°C. Alternatively, resuspend samples overnight at +2 to +8°C.

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7.2 Troubleshooting Guide, continued

Problem	Remedy
Low DNA yields	• There was an insufficient number of leukocytes in the starting sample; increase the volume of starting sample. • Adjust the volume of lysis buffer to accommodate smaller numbers of leukocytes or smaller volumes of blood. • Incomplete white blood cell lysis: see recommendations above (under "Incomplete white blood cell lysis"). • If using blood stored for 7 days at +2 to +8°C or ≤1 month at –15 to –25°C, the expected yields will be 10–15% lower than those of freshly isolated blood.
DNA is not functional in subsequent applications (*e.g.*, the A_{260}/A_{280} ratio is too high or too low).	• $A_{260}/A_{280} < 1.6$: Protein contamination is present. Follow recommendations above (under "No protein precipitation"). Check to make sure the DNA is completely in solution. • $A_{260}/A_{280} < 2.0$: RNA contamination is present. Repeat RNase treatment, and reprecipitate the DNA. Increase the incubation time for RNase treatment from 15–30 min at 37°C. Quantify the DNA prior to initiating subsequent applications. Use the same amount of DNA per application as you would typically use of DNA prepared with an alternative purification method.

*Available from Roche Applied Science

‡ Purchase of this product is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process for life science research in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Applied Biosystems or as purchased, i.e., an authorized thermal cycler.

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